



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁴ : C12N 15/00	A1	(11) International Publication Number: WO 89/00199 (43) International Publication Date: 12 January 1989 (12.01.89)
(21) International Application Number: PCT/US88/02265 (22) International Filing Date: 6 July 1988 (06.07.88) (31) Priority Application Number: 069,653 (32) Priority Date: 6 July 1987 (06.07.87) (33) Priority Country: US (71) Applicant: LOUISIANA STATE UNIVERSITY AGRICULTURAL AND MECHANICAL COLLEGE [US/US]; 155 T. Boyd Hall, Baton Rouge, LA 70803 (US). (72) Inventors: JAYNES, Jesse, M. : 623 College Hill Drive, Baton Rouge, LA 70808 (US). ENRIGHT, Frederick, M. : 5238 North Chalet Court, Baton Rouge, LA 70800 (US). WHITE, Kenneth, L. : 5737 Holly Springs Drive, Baton Rouge, LA 70817 (US).		(74) Agents: DRY, Elton, N. et al.; Pravel, Gambrell, Hewitt, Kimball & Krieger, 1177 West Loop South, 10th Floor, Houston, TX 77027 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FI, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: THERAPEUTIC ANTIMICROBIAL POLYPEPTIDES, THEIR USE AND METHODS FOR PREPARATION (57) Abstract A novel class of antimicrobial agents for animal species including cecropins, attacins, lysozymes, phage derived polypeptides, such as those transcribed from gene 13 of phage 22, an S protein from lambda phage, and an E protein from phage PhiX174, as well as, synthetically derived polypeptides of similar nature. The antimicrobial agents can be used to treat microbial infections and as components of medicinal compositions. The genes encoding for such antimicrobial agents can be used to transform animal cells, especially embryonic cells. The transformed animals including such antimicrobial cells are also included.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GA	Gabon	MR	Mauritania
AU	Australia	GB	United Kingdom	MW	Malawi
BB	Barbados	HU	Hungary	NL	Netherlands
BE	Belgium	IT	Italy	NO	Norway
BG	Bulgaria	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali		
FR	France				

-1-

THERAPEUTIC ANTIMICROBIAL POLYPEPTIDES, THEIR USE AND METHODS
FOR PREPARATION

Background of the Invention

This invention relates to certain therapeutic polypeptides useful in man and animals. Specifically, the therapeutic polypeptides are useful in cases of intra- and extracellular bacteria, fungi and protozoa which are either resistant to conventional antibiotics, difficult to treat without harm to the host cells, associated with severe infections, or connected with cases of traumatized or immune compromised hosts. In view of the properties of the therapeutic polypeptides of this invention which include definite antibacterial, anti-fungal and anti-protozoan activity, the therapeutic polypeptides provided herein are termed antimicrobial polypeptides.

Further, this invention relates to biosynthetic processes affording the antimicrobial polypeptides, including cloning or producing the desired polypeptides in various media, both as active antimicrobial polypeptides and also in the pro-form or the inactive antimicrobial polypeptide form with subsequent activation procedures. Included in these processes and procedures for biosynthesis of the antimicrobial polypeptides of this invention are novel forms of the polypeptides themselves, synthetic or semi-synthetic polypeptides and cells containing such novel polypeptides which are themselves novel compositions, or cell lines, or hybridoma.

Still further, this invention relates to novel cells, including animal cells, and particularly mammalian cells, containing such antimicrobial polypeptides, including various specific mammalian cell types which contain the antimicrobial

SUBSTITUTE SHEET

-2-

polypeptid s. Methods for treating mammals infected with certain bacteria, protozoa, or fungi, and which are resistant to known antibiotics or which are difficult to treat with such antibiotics are also included in the present invention. As a novel composition, the present invention includes specifically mammalian cells having genes encoding for such antimicrobial polypeptides; particularly, various specific embryonic cells having the genes encoding for the antimicrobial polypeptides are included in the present invention. Additionally, processes for and methods of preparing mammalian cells including the genes encoding for antimicrobial polypeptides of this invention using recombinant DNA techniques are a part of the present invention.

A number of the antimicrobial polypeptides have been found to be useful when the genes encoding therefor are incorporated into various plant species. Particularly, when introduced into the plant genome by means of Agrobacterium, the antimicrobial polypeptide encoding genes produce plant species much more resistant to certain bacterially induced disease conditions and plant pathogens. Such antimicrobial polypeptides and the incorporation of the genes encoding therefor are more fully described in U.S. patent application Serial No. 889,225, filed July 25, 1986, to Jaynes et al, which is incorporated herein by reference as if fully set forth.

Although antimicrobial polypeptides of the type envisioned in the present invention are known from the humoral response to bacterial infection of the Hyalophora cecropia (a species of large silk moth), prior to the discovery of Jaynes et al, supra, it was unknown that such antimicrobial polypeptides were useful against plant pathogens or how the same could be transformed into plants. It is likewise

SUBSTITUTE SHEET

-3-

previously unknown before the present invention, that the antimicrobial polypeptides would be similarly effective in animal, and particularly mammalian species, against certain microorganisms, or what method could be employed to transform cells of such species therewith.

It is known that certain polynucleotide molecules can be expressible in a given host and have the sequence araB promoter operably linked to a gene which is heterologous to such host. The heterologous gene codes for a biologically active polypeptide. A genetic construct of a first genetic sequence coding for cecropin operably linked to a second genetic sequence coding for a polypeptide which is capable of suppressing the biological effect of the resulting fusion protein towards an otherwise cecropin-sensitive bacterium, International Patent Publication WO86/04356, July 31, 1986.

It is therefore an object of the present invention to provide antimicrobial polypeptides for therapeutic treatment of pathogens in mammals including bacteria, fungi, and protozoa. A further object is to provide a method for treatment of man and animals having a bacterial infection with an antimicrobial polypeptide for such infection. A still further object of the present invention is to provide for a biosynthetic process to produce such antimicrobial polypeptides. Another object of the present invention is to provide for novel synthetic and semisynthetic antimicrobial polypeptides produced by recombinant DNA procedures. A still further object of the invention provides cells from a host animal transformed by genetic sequences incorporated into the cells, and ultimately to provide transformed animals which are as a result resistant to a number of pathogenic microorganisms.

SUBSTITUTE SHEET

-4-

These and still other objects of the invention are provided according to the present invention as described in the following specification.

The Invention

The present invention provides for a method for treatment of an animal having a bacterial infection caused by either or both gram-positive or gram-negative bacteria, such as by a member of the group consisting of *Brucella*, *Listeria*, *Pseudomonas* (other than *P. solanacium*), *Staphylococcus* or a protozoan infection caused by a member of the group consisting of *Trypanosoma* and *Plasmodia*, which method comprises administration to said mammal of an antibacterial amount of an antimicrobial polypeptide selected from the group consisting of a cecropin, an attacin, a lysozyme, a polypeptide transcribed from gene 13 of phage P22, an S protein from lambda phage, and an E protein from phage PhiX174. As another aspect of the present invention, there is provided a biosynthetic method for producing the antimicrobial polypeptides of the present invention which method includes the steps of

(a) microinjecting a phosphate buffered solution of genes encoding for and an antimicrobial polypeptide into selected cells, and

(b) culturing the cells to produce such antimicrobial polypeptide included in the genome of said cells.

In a still further aspect, precursor polypeptides for the antimicrobial polypeptides of the present invention are produced by a process which comprises the steps of:

(a) augmenting at the 5' end in the correct reading frame the genes encoding for the beta-lactamase gene of *E. coli* by fusing thereto genes encoding for at least one antimicrobial polypeptide free from internal methionine

3,307,101 3/77

-6-

leucine for valine or vice versa in 4 occurrences, for an internal methionine, and in the last 5 amino acids on the carboxy end. A semisynthetic cecropin having two additional amino acids, which are methionine and proline, added to the amino end of cecropin B and free from internal methionine, that is, preferably substituted with valine for the internal methionine of natural cecropin B, has shown good antimicrobial activity. The amino acid sequence for the cecropins and selected examples of other antimicrobial polypeptides of the present invention is shown in Table 1.

SUBSTITUTE SHEET

TABLE 1

CECROPIN C-37	MetProLysTrpLysValPheLysLysIleGluLysValGlyArgAsnIleArgAsnGlyIleValLysAlaGlyProAlaIleAlaValLeuGlyGluAlaLysAlaLeuCONH ₂
NATURAL	
CECROPIN B	LysTrpLysValPheLysLysIleGluLysMetGlyArgAsnIleArgAsnGlyIleValLysAlaGlyProAlaIleAlaValLeuGlyGluAlaLysAlaLeuCONH ₂
NATURAL	
CECROPIN A	LysTrpLysLeuPheLysLysIleGluLysValGlyGlnAsnIleArgAspGlyIleIleLysAlaGlyProAlaValAlaValGlyGlnAlaThrGlnIleAlaLysCONH ₂
PhIX174	
E PROTEIN-35	MetValArgTrpThrLeuTrpAspThrLeuAlaPheLeuLeuLeuSerLeuLeuLeuProSerLeuLeuIleMetPheIleProSerPheLysArgProVal
P22 P13---	36 MetLysLysMetProGluLysHisAspLeuLeuThrAlaMetMetAlaAlaLysGlnGlnGlyIleGlyAlaIleLeuIlePheAlaMetAlaTyrLeuArgGlyArg
LAMBDA S	
PHAGE PROTEIN	MetLysMetProGluLysHisAspLeuLeuAlaAlaIleLeuAlaAlaLysGlnGlnGlyIleGlyAlaIleLeuAlaPheAlaMetAlaTyrLeuArgGlyArg
MELLITIN	GlyIleGlyAlaValLeuLysValLeuThrThrGlyLeuProAlaLeuIleSerTrpIleLysArgLysArgGlnGln

-8-

Also shown in Table 1 is an antimicrobial polypeptide showing the first 35 amino acids transcribed from gene 13 of the phage P22. This phage is described in Rennell et al, Virology, 143, 280-289 (1985). A sample of the bacterium described therein was obtained from the author for use in this invention and propagated according to the directions provided. This reference is incorporated herein by reference as if fully set forth.

The S protein from Lambda phage is similar to that from phage P22, but has one less lysine unit at the amino end. A computer database search in Genbank was used to provide the DNA sequence for the S protein and the polypeptide sequence was derived by computer using DNA Inspector II.

Although smaller in amino acid chain length than the cecropins, the effective antimicrobial activity of the first 30 amino acid units of the E protein from phage PhiX174 is similar in scope. The E protein from phage PhiX174 is described by Buckley et al, Molecular General Genetics, Vol. 204, 120-125 (1986), which is incorporated herein by reference as if fully set forth.

The above described polypeptides have utility as therapeutic agents or antimicrobials for a wide variety of disease states and microbial conditions, including bacterial infections, certain fungal infections and some protozoal infections investigated. Of considerable interest are disease states which have long resisted treatment efforts, which are particularly serious, which result from both intracellular and extracellular organisms causing chronic debilitating etiologies, or which are associated with traumatized or immune compromised hosts. Specific bacterial examples are Brucella abortus, Listeria monocytogenes, Pseudomonas aeruginosa, and Staphylococcus aureus.

SUBSTITUTE SHEET

-9-

The in vitro effectiveness of the antimicrobial polypeptides is shown by simple exposure tests which provide a crude, but accurate, indication of activity. In general, such tests are carried out by exposing a known number of viable bacteria or other microorganism to one or more of the antimicrobial polypeptides of the present invention for a given time period. Following such treatment, the diluted or undiluted bacteria or microorganisms are inoculated on solid growth media or into an appropriate culture system and incubated. The microorganisms are then enumerated by the appropriate technique and the resulting numbers compared.

Several specific examples of in vitro tests were run on E. coli and B. abortus using the following general procedure:

Known numbers of bacteria are exposed to a known concentration of a selected antimicrobial agent of this invention. This mixture and appropriate controls were incubated at 37°C for up to one hour. Following incubation, the mixtures are serially diluted and plated on an agar based growth media. The inoculated plates are incubated for 3 to 5 days at 37°C, after which the numbers of bacterial colonies are counted with the results expressed as the numbers of organisms per milliliter of the original mixture. The treated and control samples are then compared and expressed as absolute numbers of organisms or the log reductions of organisms.

A still further aspect of the present invention includes animal cells containing antimicrobial polypeptides selected from a cecropin, an attacin, a lysozyme, a polypeptide transcribed from gene 13 of phage P22, an S protein from lambda phage and an E protein from the phage PhiX174. An additional aspect of the present invention is a process for producing animal cells containing such antimicrobial

-10-

polypeptides which comprises transforming embryonic animal cells with genes encoding for such antimicrobial polypeptides using microinjection of pronuclear stage embryos. Another aspect of such a process for transformation of embryonic animal cells with genes encoding for such antimicrobial polypeptides includes transfection of early preimplantation stage embryos with retroviral vectors. Still another aspect of the present invention includes a process for producing animal cells which comprises transforming embryonic animal cells with genes encoding for such antimicrobial polypeptides using electroporation. Following each of these in vitro procedures, the transformed cells are reintroduced into the host or parent mammal for development and subsequent delivery, culturing or incubating the transformed eggs of aquatic species or fowl and hatching, as a transformed species, strain or breed having microbial resistance..

The transformation of animal cells employs cells of various types and can be used without difficulty in any cell type. Specifically, both differentiated and undifferentiated cells can be used as a cellular substrate for transformation. Typically, fibroblast cells, macrophage cells, primary cells, pluripotent embryonic stem cells, pluripotent hematopoietic stem cells, phagocytic cells, plasma cells, mast cells and carcinoma cells are useful cellular materials which can be successfully transformed to express the antimicrobial agents of this invention. The cells, once transformed by procedures described below, are then reintroduced into the host for antimicrobial activity.

According to this invention, the cells are transformed to contain one or more genes coding for polypeptide antimicrobial agents. The antimicrobial agents as contemplated by the present invention include insect-, phage-derived, and

SUBSTITUTE SHEET

-5-

c dons and having a methionine codon at the 5' end of the antimicrobial gene to produce an augmented E. coli;

(b) culturing the augmented E. coli under conditions to produce beta-lactamase modified by containing at least one said antimicrobial polypeptide;

(c) isolating the modified beta-lactamase;

(d) treating the modified beta-lactamase with cyanogen bromide to cleave the antimicrobial polypeptide; and

(e) separating the antimicrobial polypeptide from the beta-lactamase,

whereby an active antimicrobial polypeptide is formed.

The antimicrobial polypeptides of the present invention are relatively small having from about 30 to about 40 amino acids and, generally, as produced in natural settings include longer chains of amino acids, for example, of up to about 160 units in length. However, it is believed that the active portion of the naturally produced material selected for antimicrobial activity is between about 30 and about 40 amino acid units in length. Such a length is unusually small to have a widespread effect as an antimicrobial polypeptide. However, the in vitro tests, discussed below, have shown such antimicrobial activity to be present. Antimicrobial effects in insects have been noted for certain of the antimicrobial polypeptides. Specifically, the cecropins, attacins, and lysozymes were determined to be present in the humoral response to bacterial infection of the Hyalophora cecropia, as noted, supra, in the application of Jaynes et al. Cecropins, which are one example of a class of an antimicrobial polypeptide of the present invention include natural cecropin B, which has 35 amino acids, natural cecropin A which has 37 amino acids and differs from cecropin B by substituting

-11-

synthetic antimicrobial agents. Such insect-derived antimicrobial agents are known for their activity in combating diseases in certain insects caused by bacteria. The humoral response of such insects includes the production of attacins, lysozymes and cecropins as naturally expressed antimicrobial agents. The selection and application of such agents to animals and the transformation of animal cells to include genes coding for such antimicrobial agents into their genomes provides a hitherto unavailable possibility. In general, the antimicrobial agents are selected from attacins, lysozymes and cecropins. Further, phage-derived proteins have also been found to contain antimicrobial activity. Typical of such phage-derived antimicrobial agents include proteins and protein fragments containing lytic activity, such as an S protein from lambda phage, an E protein from phage PhiX174, and a protein from gene 13 of phage 22.

Cecropin polypeptides or proteins include several forms of which Cecropin A, Cecropin B, Cecropin D and a modified Cecropin B having 37 amino acids instead of the usual 35, as described supra, are preferred embodiments of antimicrobial agents with which to transform animal cells. In more preferred embodiments of the present invention, animal cell transformation is carried out by a process of treating discrete colonies of such cells or cell lines by treatment with selected chemical transformants, by treatment with a selected modified retrovirus vector, or by treatment employing electroporation.

Chemical transformation is a conventional procedure described by Wigler, M. et al, Cell 11:223-232 (1977), which is also known as DNA transfection in which the foreign gene or DNA is introduced into cells in culture as a part of a coprecipitate with calcium phosphate or dextran sulfate. The

SUBSTITUTE SHEET

-12-

successful result of such procedure provides a viable cell containing one to many copies of the new gene which continuously expresses the new genetic information. Although less efficient than newer techniques, only one in a thousand (and typically one in a million) cells incorporate the new gene, DNA transfection provides a means for insertion of the antimicrobial agents of the present invention in the situation where the cell quantity is not limited.

Insertion of foreign DNA into cells by modified retrovirus vectors is a more recent development. As described by Gilboa et al, BioTechniques, Vol. 4, No. 6 (1986), pages 504-511, the technique provides for transferring a desired gene to a large fraction of a given cell population and is also referred to as retroviral-mediated gene transfer. Retroviruses have their viral genes encoded in RNA rather than DNA. When a virus penetrates a cell, the viral RNA is first converted to DNA, the DNA enters the nucleus and integrates randomly into a chromosome. The viral genes are expressed from the integrated provirus and progeny viruses are formed and leave by budding from the cell membrane. The antimicrobial agent genes of this invention are inserted into the retrovirus replacing the viral genes and using the viral integration process. A number of retrovirus vectors are useful in such a process, as described in Gilboa et al, supra. Typical are the retrovirus vectors identified as and selected from N2, N4, SAX, Mo+Py and M-MuLV. The foregoing is only an illustrative list which is non-limiting. Additional retroviral vectors will be identified and considered useful, as understood by skilled practitioners.

The technique of electroporation, well known and used primarily in plant cell work, has also been applied to animal cells recently. The electroporation process depends on the

ABSTRACT SHEET

-13-

discovery that in culture medium containing DNA fragments or genes desired for insertion, the application of an electric field causes the cells to become more porous to entry of the foreign genes, some of which will be incorporated into the cell genome and express the gene products. In a culture containing 1×10^6 to 10^7 cells, about one cell in a thousand actually incorporate the foreign DNA or genes, providing a sufficient concentration of the desired genes is present. Using a selectable marker aids identification and separation of cells incorporating the desired DNA for subsequent use.

Another aspect of the present invention is a process for providing animals with resistance to microbial infection or disease by introducing transformed animal cells of this invention into the animal. The transformed animal cells contain one or more genes coding for one or more antimicrobial agents selected from a cecropin, an attacin, a lysozyme, an S protein from lambda phage, an E protein from phage PhiX174, and a protein produced by gene 13 of phage 22. The means, procedures and processes for transforming animal cells are as described above. Further, the types of cells transformed are also described above. In general, the process is applicable to any animal subject to microbial infection or diseases with which the antimicrobial agents of this invention deal effectively. Typically, only domesticated animals of economic significance are of interest with regard to this process. However, for the prevention of contagious infection or disease, practically any animal is included within the scope of the invention. Practically, however, only those animals which are readily available for introduction of transformed cells, e.g., animals which are kept in confined areas or regularly gathered for inspection, treatment, marketing and the like, are more easily treated. Specifically, animals

SUBSTITUTE SHEET

-14-

selected from cattle, horses, pigs, sheep, goats, dogs and cats are included in the animals amenable to the present process. Fowl such as ducks, geese, chickens, turkeys and the like are also included. Additionally, fish and other marine animals or aquaculturally produced species are included.

In one aspect of the invention, the process includes introducing into the animal transformed cells to provide cells which fight certain infections or disease states by expressing the antimicrobial agents. Methods for obtaining transformable cells from a live animal are known and conventional. These methods include surgical procedures, bone marrow collection and transfer, biopsies, and the like. The cells are then transformed according to the processes described herein and reintroduced to the animal using conventional surgical or other techniques mentioned above. The cells are not rejected because they are the animal's own cells, but the stress of the gathering, transformation or reintroduction may result in a significant number of non-viable transformed cells or problems with the host animal's implantation site. The process of this aspect of the invention can be used as a treatment process or alternatively as a prophylactic procedure for preventing disease or infection to exposed animals.

Although the previously described process of transformation of animal cells includes embryos, the present invention particularly includes preimplantation stage embryos, and in certain cases, but not preferably, implanted embryos, containing in their genomes one or more genes coding for one or more antimicrobial agents selected from the group consisting of cecropins, attacins, lysozymes, an S protein from lambda phage, an E protein from phage PhiX174 and a protein produced by gen 13 of phage 22. The embryos of particular interest to this feature of the invention are

SUBSTITUTE SHEET

-15-

preimplantation stage embryos selected from, but not limited to, zygotes or one-cell embryos, 2-cell embryos, 4-cell embryos, 8-cell embryos, morulae and blastocysts, including hatched blastocysts. The transformation of such embryos with one or more genes coding for one or more antimicrobial agents of this invention is accomplished by processes described above, e.g., transfection of the embryos with retroviral vectors carrying one or more genes for the antimicrobial agents, electroporation of the embryos, treatment of the embryos with a chemical transformant solution containing one or more genes coding for said antimicrobial agents, and microinjection of the embryos with one or more genes coding for the antimicrobial agents. Microinjection is also a known and conventional procedure. However, it is highly specific and requires each embryo to be treated individually. In contrast, retroviral vector procedures treat a number of embryos at a time. The DNA transfection or chemical transformation procedures are useful, but require a greater number of embryos in order to be effective. Thus, microinjection, retroviral vectors or electroporation are preferred transformation processes for embryos because the success ratio is higher than for other processes and fewer embryos are required to accomplish a desired result. Of course, the feature of transforming embryos by the process of this invention is that the genes enter the embryonic genome and are replicated with the growing post-implant zygote which eventually results, after gestation and birth or incubation or culturing and hatching, in a transformed animal having one or more of the antimicrobial agents expressed in its genes and which can pass these characteristics onto its progeny. Thus, a further aspect and feature of the present invention is an animal having in its genome one or more genes coding for one

-16-

or more antimicrobial agents selected from the group consisting of cecropins, attacins, lysozymes, an S protein from lambda phage, an E protein from phage PhiX174, and a protein produced from gene 13 of phage 22, such that the animal is resistant to microbial diseases affected by the antimicrobial agent, especially such agents as are included in the animal's genome.

Preferably, transformed animals are selected, as before,
from cattle, horses, pigs, sheep, goats, dogs, cats, fowl,
fish, and aquacultural species, although other animals
including those raised for fur, hides, exhibition,
entertainment and observation, or simply species preservation,
can likewise become transformed animals according to this
invention. Preferably, animals of economic importance are
transformed and are included in this feature of the invention.

The transformed animals of the present invention are
resistant to a number of disease- and infection-causing
microorganisms, including bacteria, fungi and protozoa.
Specifically, microbes selected from the general classes of
Brucella, Listeria, Pseudomonas, Staphylococcus, Trypanosoma
and Plasmodium are included in the group to which the
transformed animals of the present invention are resistant.
More specifically, the transformed animals are resistant to
microorganisms selected from Brucella abortus, Plasmodium
falciparum, Trypanosoma cruzi, Listeria monocytogenes,
Pseudomonas aeruginosa, Staphylococcus aureus, and the like.
Each of the transformed animals has in its genome one or more
genes which express one or more antimicrobial agents as
described hereinabove for the specific genes coding for the
antimicrobial agents.

In a still further aspect and feature of the present
invention is a method of treatment for animals which are not

-17-

transformed includes direct injection intramuscularly, intraperitoneally, subcutaneously or intravenously of one or more antimicrobial agents of this invention in an antimicrobially effective amount. Preferably, the antimicrobial agent in unit dosage form includes an acceptable pharmaceutical carrier. Typical examples include unit dosage forms which range in concentration from about 10 to about 150 milligrams of antimicrobial agent per kilogram of animal body weight, preferably from about 11 to about 110 mg per kg. Specifically, when cecropin is the antimicrobial agent, a unit dosage amount includes about 10 to about 150 milligrams of cecropin per kilogram of body weight for the animal, preferably from about 11 to about 110 mg per kg.

Specifically, the diseases against which such treatment effectively prevents or mitigates the severity of infection or disease include those referred to hereinabove. A preferred method of treatment of animals for microbial infections or diseases comprises administering to such animals an antimicrobially effective amount, as defined hereinabove, of one or more antimicrobial agents selected from the group consisting of a cecropin, an attacin, a lysozyme, an S protein from lambda phage, an E protein from phage PhiX174, and a protein produced by gene 13 of phage 22. Typically and more preferably, the antimicrobial agent is a cecropin and is selected from Cecropin A, Cecropin B, Cecropin D, or the like. Most preferred of the cecropins is a modified cecropin having 37 amino acids with a methionine and proline added at the amino end and no internal methionines. The preferred amino acid sequences of typical useful polypeptide antimicrobial agents are given in Table 1 above.

Another feature of the present invention provides a medicinal composition which includes, in various formulations

SUBSTITUTE SHEET

-18-

and with various pharmaceutical carriers, a unit dosage form of the antimicrobial agents of this invention which are selected from a cecropin, an attacin, a lysozyme, an S protein from lambda phage, an E protein from phage PhiX174, and a protein produced by gene 13 of phage 22. Acceptable pharmaceutical carriers include water, alcohol, solvents and oils in the form of aromatic waters, liquors, solutions, tinctures, elixirs, spirits, parenteral solutions, physiologically buffered media, and the like. The unit dosage form of medicinal composition can be used for treating, humans and animals, including those described above. Unit dosage forms have the same amount of antimicrobial agent as disclosed for the method of treatment or some fractional equivalent thereof to provide a treatment regimen employed over a period of time. The optimum upper and lower therapeutic amounts and any contra-indications have not yet been fully established.

The safety of antimicrobial agents according to the present invention was tested according to standard procedures in animal studies. The procedures and results are given in the following Example 1.

EXAMPLE 1

Part A

Six BALB/C mice, 4 to 5 weeks of age, maintained on commercial rodent ration fed ad libidum and in general good health were each inoculated with 1.76 milligrams/day of cecropin C-37 in balanced salt solution intramuscularly for 4 consecutive days. No other change was made in diet or conditions. The mice were observed twice daily and no adverse reactions were noted. At the end of the fourth day, three of the nine were humanely destroyed and examined. After 30 days,

SUBSTITUTE SHEET

-19-

3 of the 6 were given an additional 1.76 milligrams each. No adverse reactions were noted.

The remaining mice were all killed 7 days after the last inoculation. Examination of organs and tissues indicated no gross pathological changes were present in the organs or at the injection sites. None of these mice produced detectable levels of IgG antibody to the antimicrobial agent.

Part B

Following the procedure above in Part A, three BALB/C mice were given 110 milligrams/kilogram of body weight of cecropin C-37 injected intramuscularly in balanced salt solution for 6 days. White cell counts and differentials were performed on a daily basis. Ten days after the last inoculation, the mice were again injected intramuscularly with 110 milligrams/kilogram of body weight with Cecropin C-37. Observation revealed no adverse effects during the procedure. All three mice were killed 7 days after the final inoculation and tissues were examined. All mice had enlarged spleens but were otherwise unremarkable. No Cecropin C-37 antibodies were detected.

The method of treatment of an animal employing the antimicrobial agents of this invention are illustrated by the following Examples.

EXAMPLE 2

A total of 18 BALB/C mice, maintained on commercial rodent ration fed ad libidum, 4 to 5 weeks of age and in good health were inoculated intraperitoneally with 3 to 5×10^8 Brucella abortus in physiological saline. On the 12th day post infection, 6 of the mice were each inoculated intramuscularly with 0.176 milligrams/day of cecropin C-37 antimicrobial agent in balanced salts, 6 were likewise treated

SECRET

-20-

with 0.176 milligrams/day of tetracycline and 6 were likewise given sterile water, all for a period of 4 days. Then, nine mice were sacrificed on the 16th day post infection and spleen tissue examined morphologically, histologically and by culturing according to standard procedures for Brucella abortus. The concentrations found are given in the following table.

TABLE 2

Concentration of Brucella abortus in BALB/c Mouse Spleen Tissue After 4 Days Treatment (per gram of spleen)

<u>Control</u>	<u>Tetracycline</u>	<u>Cecropin C-37</u>
8.15x10 ³	4.4x10 ³	1.59x10 ³

The remaining 9 mice were sacrificed on the 23rd day post infection and examined in the same manner and no difference in result was observed. This result indicates that the antimicrobial agent of this invention is capable of significantly decreasing the level of infection and growth of Brucella abortus in mice.

In vitro evaluation of the antimicrobial agents of this invention were carried out by the following general procedure. A known number of viable microorganisms were exposed to a known concentration of the antimicrobial agent for a given time period. Following exposure, the undiluted or diluted microorganisms were plated on solid growth media, e.g., agar plates. The plates were incubated and numbers of organisms determined by making plate counts. Comparisons are then made with the non-treated bacteria for evidence of inhibition, mortality, or both. The following Examples give detailed experimental procedures and results for several microorganisms and antimicrobial agents.

-21-

EXAMPLE 3

To a suitable container was added 50 microliters of the bacteria under investigation, in this instance, E. coli E/2/64, obtained from Cornell University, and 50 microliters of phosphate buffered saline solution (PBS), prepared from NaCl - 80.0g, KCl - 3g, Na₂HPO₄ - 0.73g, KH₂PO₄ - 0.2g, with dilution of 10 milliliters of mixture with 90 milliliters of water and sterilize by autoclaving. The E. coli is collected in late log phase growth at which the majority of cells are in growth phase at 18-24 hours. In addition, the same mixture was prepared with 50 microliters of natural cecropin B or modified cecropin C-37. The mixtures were incubated at room temperature for 1 hour, and then at 37°C for 30 minutes. The mixtures were diluted to 10⁻¹ or 10⁻³ with serial dilutions at 7 drops per dilution (10 microliters per drop), plated on agar growth media, which is a tryptose agar. The plates were incubated for 3 days at 37°C. Plate counts were then made and the results given below.

TABLE 3

Effect of Antimicrobial Agent on E. coli

<u>Antimicrobial Agent</u>	<u>Concentration</u>	<u>Results</u>
Cecropin B	5x10 ⁻⁶ molar	no growth
Cecropin C-37	5x10 ⁻⁶ molar	no growth

Combinations of both natural cecropin B and cecropin C-37 with lysozyme (25:25 microliter mixture) also prevented growth at 10⁻⁷ molar concentrations, but lysozyme itself at concentrations of 10 micrograms, 1 milligram, and 10 milligrams per milliliter all showed positive growth of E. coli at both 10⁻¹ and 10⁻³ dilutions. Further, lower

SUBSTITUTE SHEET

-22-

concentrations of 1 nanomolar and 1 micromolar of both cecropin species showed positive E. coli growth.

EXAMPLE 4

The procedure of Example 3 was followed, except the microorganism was Brucella abortus, isolated by conventional procedure. The results are shown in Table 4 below.

TABLE 4

Effect of Antimicrobial Agent on Brucella abortus

<u>Antimicrobial Agent</u>	<u>Concentration</u>	<u>Results</u>
Cecropin B	5×10^{-5} molar	no growth
Cecropin C-37	5×10^{-5} molar	no growth

Combinations of modified Cecropin C-37 and natural cecropin B with lysozyme at 10 micrograms, 1 milligram and 10 milligrams per milliliter were effective to prevent growth only at the lowest concentration of lysozyme with cecropin C-37; otherwise, some growth of the B. abortus was noted.

The effect of antimicrobial agents of the present invention on Trypanosoma cruzi was investigated and the results and procedure are given in the following Example(s).

EXAMPLE 5

When Vero cells were infected on a 1:1 ratio with Trypanosomes which had been treated by soaking in a 100 micromolar solution of Cecropin B modified (C-37) antimicrobial agent in balanced salt prior to the infection process, the Vero cells had no infection with Trypomastigotes compared to 15 percent infection with a non-treated controls sample. The cecropin C-37 was completely effective in destroying the pathogen.

-23-

The effect of the antimicrobial agents of this invention on the malarial agent Plasmodium falciparum is illustrated in the Example below. Plasmodium falciparum attacks human red blood cells and remains in them. Because the red cell has no DNA itself, when the P. falciparum-containing red blood cell is treated with tritiated hypoxanthine, the radioactively labeled compound will be taken up by the live malarial parasite's DNA. Thus, radiation counts from the labeled H^3 will indicate the degree to which the antimicrobial agent successfully destroys the microorganism, i.e., the higher the radiated counts, the more viable P. falciparum extant.

EXAMPLE 6

Monolayer culture of Vero cells, derived from African Green Monkey kidney cells and available commercially, are infected by trypomastigote stage of Trypanosoma cruzi. The number of infected cells are determined by counting the amastigotes which develop intracellularly following penetration by the parasites. The cells were grown in microscope slide chambers at a density of about 100,000 cells per cubic millimeter. After a period of exposure by Trypanosoma cruzi to the Vero cells at a 1:1 ratio, several of the chambers were treated with Cecropin C-37 at a concentration of 100 micromolar. The slide chambers were fixed with formalin at various periods, usually 48, 72 and 95 hours post-infection, and then stained with geimsa. Random counts were made along a line down the microscope slide until several hundred cells were counted in replicates per slide. The results in numbers of amastigotes infecting the cells over time are given in the Table below.

-24-

TABLE 5

Effect of Antimicrobial Agent on Trypanosoma cruzi

Hours Post- Infection	No. of Amastigotes per 100 Mammalian Cells		Average No. of Amastigotes per Infected Cell		% of Cells Infected w/ Amastigotes	
	Control	Treated	Control	Treated	Control	Treated
48	20	15	3	3	15	14
72	50	5	4	3	16	2.5
96	225	0	17	1.5	14	0

EXAMPLE 7

To four 150 ml culture flasks containing 50 ml of human red blood cells was added 0.5 weight percent of Plasmodium falciparum microorganisms in RPMI media. The mixture was cultured for a week under the conditions of 37°C and CO₂ incubator. Then, with one flask not having anything added and serving as a control, varied amounts of a cecropin C-37 and 10⁻⁵ molar solution of hypoxanthine containing 50 microcuries of tritiated hypoxanthine in the same media solution was added. The concentrations of experiments carried out included 1 micromolar, 20 micromolar and 200 micromolar amounts of cecropin C-37. After 24 hours at culture conditions, the solution was filtered off. The filter cake was measured with a liquid scintillation counter to determine the amount of uptake by the DNA of P. falciparum. The results are shown in Table 6 below.

The experiments were repeated at lower levels of infection with P. falciparum, i.e., 0.25 weight percent, 0.125 weight percent and 0.0625 weight percent. The results are also shown in Table 6.

SUBSTITUTE SHEET

TABLE 6

Uptake of Tritiated Hypoxanthine by
Plasmodium falciparum

Concentration of Antimicrobial (micromoles)	0	1	20	200
For Cecropin C-37 Percent Infection	Counts per Minute x 10 ⁻¹			
0.5	2720	2350	1925	60
0.25	1200	1053	946	40
0.125	520	470	435	30
0.0625	340	315	295	20
For Cecropin C-35 Percent Infection				
0.5	2730	2860	2333	50
0.25	1333	1293	1066	40
0.125	625	565	495	20
0.0625	395	380	405	25

The results shown in the Table above indicate that as the concentration of antimicrobial agent increases from 0 (the control) to 200 micromolar, the counts per minute decrease, in most instances even at low concentrations and dramatically at higher concentrations. The lower counts indicate that the pathogenic microorganism is not taking up the tritiated hypoxanthine and the reason for this is that the antimicrobial agent is having an adverse affect on the pathogenic microorganism.

The effect of the antimicrobial agents of this invention on yeast is illustrated in the following example.

EXAMPLE 8

The yeast Saccharomyces cerevisiae was grown to late log phase in nutrient broth containing 10 grams tryptose and 5 grams of yeast extract plus 2 weight percent glucose. It was

-26-

then diluted to about 500,000 cells per milliliter in 0.01M phosphate buffer solution at pH 6.8. Then the appropriate antimicrobial agents, controls or previously known lysing agents were introduced at molar concentrations indicated in the Table below and the resultant solutions incubated at 37°C for 1 hour. The solutions were diluted 1000 fold and plated on nutrient agar plus glucose. The next day, plate counts were taken and the level of surviving yeast determined. The results are given in the Table below. The control was an unrelated peptide of 15 amino acids synthesized in a manner similar to that used to produce cecropin 37. Mellitin is a natural cell lysing agent and was used to indicate the best condition.

TABLE 7

Effect of Antimicrobial Agents on Yeast (Saccharomyces cerevisiae)

<u>Antimicrobial Agent</u>	Number of Yeast Cells After Treatment (x 1000)			
	<u>Control</u>	<u>Mellitin</u>	<u>Cecropin</u>	<u>Cecropin 37</u>
<u>Molar Concentration of Antimicrobial Agent</u>				
0	500	500	500	500
5×10^{-6}	500	260	500	400
5×10^{-5}	500	0	75	75
7.5×10^{-4}	500	---	0	0

Although the antimicrobial agents of the present invention require a slightly higher concentration, they have an effect similar to a known lysing agent for yeast cells.

EXAMPLE 9

In a manner similar to Example 8 above, about 250,000 cells of E. coli was treated with each of the antimicrobial

-27-

agents at various concentrations. The results are shown in Table 8 below.

TABLE 8

Effect of Antimicrobial Agents on *E. coli*

<u>Molar Concentration of Antimicrobial Agent</u>	<u>Control</u>	<u>Mellitin</u>	<u>Cecropin</u>	<u>Cecropin 37</u>
0	260	260	260	260
6×10^{-6}	260	20	10	10
2.5×10^{-4}	333	10	10	10

EXAMPLE 10

In a manner similar to Example 8 above, several human and animal pathogenic bacteria were treated with an antimicrobial agent of this invention. The *Pseudomonas* and one of the *Staphylococcus* strains were antibiotic resistant strains. The results are given in the Table below.

TABLE 9

Treatment of Several Pathogenic Bacteria with Antimicrobial Agent

<u>Bacteria Type</u>	<u>Number of Bacteria Cells After Treatment (x 1000)</u>		
	<u><i>Pseudomonas</i> <i>aeruginosa</i></u>	<u><i>Staphylococcus</i> <i>intermedius</i></u>	<u><i>Staphylococcus</i> <i>intermedius</i> (antibiotic)</u>
<u>Molar Concentration of Antimicrobial Agent</u>			
0	1000	1000	1000
1×10^{-6}	10	50	40
1×10^{-5}	0	20	20
1×10^{-4}	0	0	0

SUBSTITUTE SHEET

-28-

This result indicates the effectiveness of the antimicrobial agents of this invention in destroying human and animal pathogenic bacteria in vitro.

As indicated hereinabove, the antimicrobial agents of the present invention are incorporated into mammalian embryos by electroporation, microinjection or retroviral vectors. The following procedure can be used to transform mammalian embryos by electroporation.

Various stages of preimplantation embryos from one-cell to blastocyst stage are electroporated in a Biorad electroporator using voltage of 100 to 300 volts at pulse durations of 10 to 20 microseconds, pulse frequencies of 1, 2 or 3 and at temperatures of 4, 25 and 37°C. The embryos will be placed in either 0.35 molar sucrose solution or phosphate buffered saline (PBS) solution containing from 20 to 500 micrograms of DNA per milliliter of at least one antimicrobial agent described hereinabove, and preferably several, and then treated or shocked according to the above parameters. After treatment, the embryos are left undisturbed for 5 to 10 minutes and are then removed from the treating or shocking chamber, washed in fresh medium and are transferred and implanted or placed in synchronized recipient mammals for continued gestation and birth.

In a similar manner, mammalian or animal cells are electroporated, except that a greater number of cells are available, e.g., from about one hundred thousand to about one million cells can be placed in the treating chamber of the electroporator, and an increased pulse duration of 7 milliseconds or more at voltages from 100-400 volts. Additionally, if the foreign or antimicrobial gene is fused to a marker or selectable gene, such as a neomycin-resistance gene, after electroporation the treated cells can be cultured

SECRET

in a selecti n medium in which only transformed cells expressing the f reign gen will survive. Thus, insuring the transfer to an animal of active cells containing genes expressing one or more of the antimicrobial agents.

The microinjection process can be carried out by known techniques which are described for the antimicrobial agents of the present invention as follows. The transfer of genes coding for at least one antimicrobial agent of this invention are carried out on pronuclear stage embryos. Such pronuclear stage zygotes will be recovered 12 to 50 hours after breeding, depending on the species used including cattle, goats, pigs, sheep, mice and the like. The embryos will be collected and maintained on Dulbecco's phosphate buffered saline (PBS) supplemented with pyruvate, glucose, 10 weight percent heat-treated fetal calf serum and 1 weight percent antibiotic-antimycotic. Micromanipulations are performed using a Zeiss ICM 405 inverted microscope equipped with two Leitz micromanipulators. Two pico-liters of purified gene preparation for at least one of the antimicrobial agents are injected into the male (larger) pronuclei using a beveled glass micropipette having an outside diameter of 1-3 micrometers. Approximately 60 percent of the embryos survive the required manipulations and injection.

The embryos are immediately transferred to appropriate synchronized recipients following microinjection and subsequent pregnancies are carried to term.

The resulting offspring are evaluated for incorporation of the genes coding for the antimicrobial agents of this invention using a two-phase evaluation. DNA preparations from peripheral blood lymphocytes, skin and liver biopsies are tested for incorporation of the antimicrobial agent genes by Southern blots using a specific gene prob . In the second

-30-

phase, animals testing positively by the Southern blot are challenged with various microbes to evaluate resistance. Generally, about 20 percent of microinjected offspring are transgenic.

The procedure for retroviral vector process is similar to that described by Van der Putten et al in Proceedings of the National Academy of Science of the U.S.A., Vol. 82, pages 6148-6152, September 1985, Cell Biology. According to that procedure, recombinant retroviral vector DNA was efficiently inserted into the mouse germ line via infection of preimplantation mouse embryos. The eight-cell stage embryos were flushed from oviduct-uterus junctions with modified Whittens medium, see Whitten, W.K., (1971) "Advances in Bioscience" 6, 129-139. The zona pelucida was removed using Pronase at 22 units/ml or acidified Tyrode's solution, see Nicholson, G.L. et al (1975), "Journal of Cell Biology", 66, 263-274. The embryos were cultured for 16 hours on top of monolayers of virus-producing cells in the presence of Polybrene (see Toyoshima et al, 1969, "Virology", 38, 414-436) at 4 micrograms per milliliter at 37°C in Dulbecco's modified Eagle's medium plus 10 (v/v) percent fetal calf serum in 5 percent CO₂ in air. After infection, morulae were cultured about 2 to 4 hours in modified Whitten's medium under a layer of equilibrated paraffin oil (see Hoppe, P. et al, 1973, "Biological Reproduction", 8, 420-426) before transfer into hours of pseudopregnant females. Subsequent testing of the offspring, carried out as described above, evaluate the efficiency of transfer of the genes coding for antimicrobial agents of this invention.

More specifically, as used in the process of the present invention, the retroviral vector process for transformation of specific animals includes the incubation of a confluent

SUBSTITUTE SHEET

Chemical transformation of embryonic animal cells according to this invention can be accomplished by calcium phosphate-mediated uptake using the procedure demonstrated by Wigler et al, supra, which is incorporated herein by reference as if fully set forth. This procedure is used in general to insert into the genome of mammalian cells growing in culture a fragment of DNA carrying one or more genes, in this instance, one or more genes coding for the antimicrobial agents described above.

... ..

-32-

if transfection can be increased by using diethylaminoethyl dextran or dextran sulfate instead of calcium phosphate or shocking the embryonic cells with glycerol after 2 hours of incubation. Under optimal conditions one embryonic cell in 100 to 1000 can be obtained which has integrated and expressed the antimicrobial genes. A selectable marker, included with the antimicrobial genes can be used to increase the usual 10^{-5} to 10^{-7} efficiency of chemical transfection using; for example, a mutant dihydrofolate reductase gene which protects the embryonic cells from methotrexate, provides a successful selector for embryonic cells incorporating the exogenous antimicrobial genes. Advantageously, chemical transformation techniques are simple to perform, require no special equipment and involve no infectious agent, as in retroviral transfection; but multiple copies, usually in head to tail relation, are transferred and the efficiency for embryonic cells, which are numerically low, is lower.

One method of obtaining the antimicrobial agents of the present invention is illustratively described using cecropin as a model. However, the general procedure would be applicable to any of the antimicrobial agents useful in the present invention. The modified cecropin C-37 gene has been synthesized by a DNA synthesis machine as shown below:

```
GATCTATGCCGAAATGGAAAGTCTTCAAGAAAATTGAAAAAGTCGGTCGCAACATTC
  ATACGGCTTTACCTTTCAGAAAGTTCTTTTAACTTTTTCAGCCAGCGTTGTAAG
GAAACGGTATTGTCAAGGCTGGACCAGCGATCGCGTTTTTAGGCGAAGCCAAAGCGC
CTTTGCCATAACAGTTCCGACCTGGTCGCTAGCGCCAAAATCCGCTTCGGTTTCGCG
TAGGATAA
ATCCTATTCTTAA
```

The above gene sequence was programmed into a DNA synthesizer commercially available from Applied Biosystems and

SUBSTITUTE SHEET

-33-

6 DNA fragments were produced using the triester method as described by Ito et al, Nucleic Acids Research, Vol. 8, 5491 (1982). The fragments were treated with polynucleotide kinase to attach a phosphate group at the 5' end and heated for 5 minutes at 90°C. The treated fragments were mixed together in equimolar amounts in TE Buffer solution (0.01 molar Tris at pH 8 and 0.001 molar EDTA). The fragments were allowed to anneal slowly over 3 hours. Then the plasmid vector, which will allow insertion into a production site, in this instance, lambda left hand promoter plasmid, cut with BglII and EcoRI restriction enzymes, was added to the prepared fragments at 0.1 concentration based on the total DNA sequences originally employed. With the addition of T4 DNA ligase to the mixture of prepared gene sequences, the mixture was left overnight at 12.5°C and the genes were incorporated into the plasmid vector.

To check the ligation of DNA sequences and insertion into the plasmid vector with appropriate expression of the desired polypeptide, competent cells of E. coli HB101, obtained from Bethesda Research Laboratories, Bethesda, Maryland, as a frozen suspension at -70°C with dry ice, were transformed by taking 100 microliters of the HB101, thawing on ice, in the presence of 10-100 nanograms of added DNA prepared in plasmid vector form above. The mixture is allowed to stand for 30 minutes, after which it is heat shocked by heating for 30 minutes at 42°C. Then, culture media to make up to 1 milliliter is added, about 900 microliters, and the mixture is allowed to sit for 2 hours at 37°C. Then, the mixture is divided and plated out on 10 plates and grown for 1 day. The plates are then examined and viable colonies picked and screened for the proper fragments by selecting clones growing in 1 ml of culture media, lysing with detergent, precipitating

BEST COPY AVAILABLE

-34-

the plasmid, digesting with BglII and EcoRI restriction enzymes. Examination after agarose gel electrophoresis should show the plasmid and the cecropin gene upon agarose gel staining.

Once the proper clones are identified as properly sequenced, transformation into a suitable host and expression of the polypeptide useful as an antimicrobial agent can be accomplished. A polypeptide leader sequence, having the DNA polynucleotide sequence below, is fused to the cecropin gene:

GATCTATGAACTTCTCCCGTATCTTTTCTTCGTTTTCGCTCTGGTTCTGGCTTCT
ATACTTGAAGAGGGCATAGAAAAGAAGCAAAGCGAGACCAAGACCGAAGA

ACTGTTTCCGCTGCACCGGAACCGG
TGACAAAGGCGACGTGGCCTGGGCCCTAG

The fusion occurs by treating the above gene sequences with polynucleotide kinase in equimolar amounts in TE Buffer by heating at 90°C and then cooling to 70°C, then equimolar amounts of pCEC1 (the cecropin gene in a plasmid vector) cut with BglII and the mixture is allowed to anneal slowly at room temperature. Then polynucleotide ligase is added in an amount sufficient to ligate the plasmid vector with pCEC1 overnight. The ligated plasmid, hereafter identified as pCEC2 (containing the plasmid vector, the cecropin leader sequence and the synthesized cecropin gene), is then transformed with *E. coli* HB101 in the same manner as indicated hereinabove.

Appropriate clones are picked and separated, and identified as pCEC2. Then, pCEC2 is cut with BglII restriction enzyme, added to an equimolar amount of beta lactamase signal peptide also cut with BglII having the DNA sequence given below:

GATCTATGAGCATCCAGCACTTCCGTGTTGCTCTGCCGTTCTTTGCTGCTTTCTGCCTGCCG
ATACTCGTAGGTCGTGAAGGCACAACGAGACGGCAAGAAACGACGAAAGACGGACGGC

SUBSTITUTE SHEET

-35-

GGTTTTTCGCTCACCCGGAG
CCAAAAGCGAGTGGGCCTCCTAG

and, in the same manner as above, the above gene sequence is treated with polynucleotide kinase and then heated and annealed slowly at room temperature and ligating with T4 DNA ligase. The resulting solution contains a mixture in which a plasmid has the beta lactamase signal peptide sequence attached to a cecropin leader which is attached to the cecropin gene in at least some amount. This is determined by transforming again with E. coli HB101 culturing, picking suitable clones and cutting with BglII and EcoRI. The resulting mixture, which is pCEC2 which is BglII and EcoRI cut, is mixed with equimolar amounts of expression vector plasmid and pCEC2 plasmid ligated with polynucleotide ligase. The resulting plasmid mixture is transformed with E. coli HB101, suitable clones are selected and identified as having the proper cecropin secretory E. coli.

Having obtained E. coli with cecropin plasmid pCEC2 secreting properties, it is then necessary to scale up the process through a series of increasingly larger containers until thousand liter culture fermentor vessels are used and separate the desired cecropin or other antimicrobial polypeptide agent. An interim step of cyanogen bromide cleavage to obtain the active cecropin is envisioned by the process of the present invention.

Having described the present invention, one skilled in the art will readily envision changes and variations in the invention which are nevertheless within the spirit and scope thereof. Accordingly, it is desired that the present invention be limited only by the lawful scope of the following claims.

RECEIVED 1989 11 15

-36-

CLAIMS:

1. A transformed animal cell having in its genome one or more genes coding for an antimicrobial agent selected from insect and phage derived antimicrobial agents, such that said cell is capable of expressing one or more of said antimicrobial agents.
2. The transformed animal cell of Claim 1 in which said insect and phage derived antimicrobial agent expressed is selected from the group consisting of attacins, cecropins, lysozymes, an S protein from lambda phage, an E protein from phage PhiX174, and a protein produced by gene 13 of phage 22.
3. The transformed animal cell of Claim 1 which is selected from fibroblast cells, macrophage cells, primary cells, pluripotent embryonic stem cells, pluripotent hematopoietic stem cells, phagocytic cells, plasma cells, mast cells and carcinoma cells.
4. The transformed animal cell of Claim 2 in which said antimicrobial agent is a cecropin.
5. The transformed animal cell of Claim 4 in which said cecropin is selected from cecropin A, cecropin B, cecropin D and a modified cecropin B having methionine and proline added at the amino end and free from internal methionine.
6. The transformed animal cell of Claim 5 in which said cecropin is Cecropin B and said cell contains the gene sequence as follows:

SUBSTITUTE SHEET

-37-

AAATGGAAGTCTTCAAGAAAATT GAA AAA ATG GGT CGC AAC ATT CGA AAC GGT ATT GTC AAG
GCT GGA CCA GCG ATC GCG GTT TTA GGC GAA GCC AAA GCG CTA GGA TAA

7. The transformed animal cell of Claim 5 in which said antimicrobial agent is a modified cecropin B and said cell contains the gene sequence as follows:

ATGCCGAAATGGAAGTCTTCAAGAAAATT GAA AAA GTC GGT CGC AAC ATT CGA AAC GGT ATT GTC
AAG GCT GGA CCA GCG ATC GCG GTT TTA GGC GAA GCC AAA GCG CTA GGA TAA

8. The transformed animal cell of Claim 2 in which said antimicrobial agent is an acidic attacin and said cell has the gene sequence as follows:

ATG
GAC GCG CAC GGA GCC CTT ACG CTC AAC TCG GAT GGT ACC TCT GGT GCT GTG GTT
AAA GTA CCC TTT CGT GGT AAC GAC AAG AAT ATA GTA AGC GCT ATC GGT TCC GTA
GAC TTA ACT GAT AGG CAG AAA CTA GGC GCT GCA ACC GCT GGA GTG GCA CTG GAT
AAT ATA AAC GGT CAC GGA CTA AGT CTC ACG GAT ACA CAC ATC CCC GGG TTC GGA
GAC AAG ATG ACA GCA GCC GGC AAA GTG AAT GTC TTC CAC AAT GAT AAC CAC GAC
ATC ACA GCG AAG GGT TTC GCC ACC AGA AAC ATG CCG GAT ATT GCT AAT GTA CCT
AAT TTC AAG ACT GTC GGT GGC GGA ATA GAC TAT ATG TTC AAA GAT AAG ATT GGT
GCA TCT GCG AGC GCC GCT CAC ACG GAC TTT ATC AAT CGC AAC GAC TAC TCT CTT
CAC GGG AAA CTG AAC CTC TTC AAG ACT CCT GAT ACC TCG ATT GAT TTC AAC GCC
GGT TTC AAG AAG TTC GAT ACA CCT TTC ATG AAG TCC TCT TGG GAG CCT AAC TTC
GGA TTC TCA CTT TCT AAA TAT TTC TGA

9. The transformed animal cell of Claim 2 in which said antimicrobial agent is a lysozyme and said cell has the gene sequence as follows:

SUBSTITUTE SHEET

-38-

AGATCTGTTTCATG AAA CGT TTC ACG AGA TGC GGG TTA GTG CAG GAG CTT AGG AGA CGA GGC
TTC GAT GAA ACT TTG ATG AGT AAC TGG GTC TGC CTT GTC GAG AAC GAA AGC GGA CGG TTT ACC
GAT AAA ATC GGT AAA GTT AAC AAG AAC GGA TCT CGA GAC TAC GGC CTC TTC CAG ATC AAT GAC
AAA TAC TGG TGC AGT AAG GGA TCC ACT CCT GGA AAG GAT TGC AAC GTG ACT TGT AAT CAG CTA
CTG ACT GAC GAC ATT AGC GTG GCA GCT ACG TGC GCG AAG AAG ATT TAC AAA CGG CAC AAG TTT
GAC GCT TGG TAC GGA TGG AAA AAT CAG TGT CAA CAT GGA CTG CCA GAT ATT AGC GAC TGT TAG
AGACGACTTATTATAGCCTTC GTTTCATGAAAG AGATCT.

10. The transformed animal cell of Claim 2 in which said antimicrobial agent is an E protein from phage PhiX174 and said cell contains the gene sequence as follows:

ATGGT ACGCTGGACT TTGTGGGATA CCCTCGCTTT CCTGCTCCTG TTGAGTTTAT
TGCTGCCGTC ATTGCTTATT ATGTTTCATCC CGTCATTCAAACG GCCTGTCTCA
TCATGGAAGG CGCTGAATTT ACGGAAAACA TTATTAATGG CGTCGAGCGT
CCGGTTAAAG CCGCTGAATT GTTCGCGTTT ACCTTGCGTG TACGCGCAGG
AAACACTGAC GTTCTTACTG ACGCAGAAGA AAACGTGCGT CAAAATTAC
GTGCGGAAGG AGTGA.

11. The transformed animal cell of Claim 2 in which said antimicrobial agent is an S protein from lambda phage and said cell contains the gene sequence as follows:

ATGAA GATGCCAGAA AAACATGACC TGTGCGGCCG CCATTCTCGC GGCAAAGGAA
CAAGGCATCG GGGCAATCCTT GCGTTTGCAA TGGCGTACCT TCGCGGCAGA
TATAATGGCG GTGCGTTTAC AAAACAGTA ATCGACGCAA CGATGTGCGC
CATTATCGCC TGGTTCATTC GTGACCTTCT CGACTTCGCC GGACTAAGTA
GCAATCTCGC TTATATAACG AGCGTGTTTA TCGGCTACAT CGGTACTGAC
TCGATTGGTT CGCTTATCAA ACGCTTCGCT GCTAAAAAAG CCGGAGTAGA
AGATGGTAGA AATCAATAA.

SUBSTITUTE SHEET

-39-

12. The transformed animal cell of Claim 2 in which said antimicrobial agent is a protein produced by gene 13 of phage 22 and said cell contains the gene sequence as follows:

ATGAA AAAGATGCCA GAAAAACATG ATCTGTTAAC CGCCATGATG GCGGCAAAGG
AACAGGGCAT CGGGGCAATC CTCGCGTTTG CAATGGCGTA CCTTCGCGGT
CGGTATAATG GCGGTGCGTT TAAGAAAACA CTAATAGACG CAACGATGTG
CGCCATTATC GCCTGGTTCA TTCGTGACCT TTAGTCTTC GCCGGACTGA
GTAGCAATCT TGCTTACATA GCGAGTGTGT TTATCGGCTA CATCGGCACA
GACTCGATTG GTTCGCTAAT CAAACGCTTC GCTGCTAAAA AAGCCGGAGT
CGATGATGCA AATCAGCAGT AA.

13. A process for producing transformed animal cells capable of expressing one or more antimicrobial agents selected from the group consisting of cecropins, attacins, lysozymes, an S protein from lambda phage, an E protein from phage PhiX174, and a protein produced by gene 13 of phage 22 comprising transforming said animal cells with one or more genes coding for said antimicrobial agents.

14. The process of Claim 13 wherein said animal cells are selected from fibroblast cells, macrophage cells, primary cells, pluripotent embryonic stem cells, hematopoietic stem cells, phagocytic cells, mast cells and carcinoma cells.

15. The process of Claim 13 in which said animal cells are transformed by

- (a) treatment with chemical transformants,
- (b) treatment with a modified retrovirus vector, or
- (c) treatment employing electroporation.

SUBSTITUTE SHEET

-40-

16. The process of Claim 15 in which said animal cells are transformed by treatment with a chemical transformant.

17. The process of Claim 16 in which said chemical transformant is selected from calcium phosphate and dextran sulfate.

18. The process of Claim 17 in which said chemical transformant is calcium phosphate.

19. The process of Claim 15 in which said animal cells are transformed by treatment with a modified retrovirus vector.

20. The process of Claim 19 in which said modified retrovirus vector is selected from the group consisting of N2, N4, SAX, Mo+Py, and M-MuLV.

21. The process of Claim 20 in which said modified retrovirus vector is N2.

22. The process of Claim 15 in which said animal cells are transformed by electroporation.

23. The process of Claim 13 in which said antimicrobial agent is a cecropin.

24. The process of Claim 23 in which said cecropin is selected from cecropin A, cecropin B, and a modified cecropin B having methionine and proline added at the amino end and free from internal methionine.

SUBSTITUTE SHEET

-41-

25. The process of Claim 24 in which said cecropin is Cecropin B and said transformed animal cells contain the gene sequence as follows:

AAAGTCTTCAAGAAA ATT GAA AAA ATG GGT CGC AAC ATT CGA AAC GGT ATT GTC AAG GCT GGA
CCA GCG ATC GCG GTT TTA GGC GAA GCC AAA GCG CTA GGA TAA.

26. The process of Claim 24 in which said antimicrobial agent is a modified cecropin B and said transformed animal cells contain the gene sequence as follows:

ATGCCGAAATGGAAAGTCTTCAAGAAAATT GAA AAA GTC GGT CGC AAC ATT CGA AAC GGT ATT GTC
AAG GCT GGA CCA GCG ATC GCG GTT TTA GGC GAA GCC AAA GCG CTA GGA TAA.

27. The process of Claim 13 in which said antimicrobial agent is an acidic attacin and said transformed animal cells contain the gene sequence as follows:

ATG
GAC GCG CAC GGA GCG CTT ACG CTC AAC TCC GAT GGT ACC TCT GGT GCT GTG GTT
AAA GTA CCC TTT CGT GGT AAC GAC AAG AAT ATA GTA ACC GCT ATC GGT TCC GTA
GAC TTA ACT GAT AGG CAG AAA CTA GGC GCT GCA ACC GCT GGA GTG GCA CTG GAT
AAT ATA AAC GGT CAC GGA CTA AGT CTC ACG GAT ACA CAC ATC CCC GGC TTC GGA
GAC AAG ATG ACA GCA GCG GGC AAA GTG AAT GTC TTC CAC AAT GAT AAC CAC GAC
ATC ACA GCG AAG GCT TTC GCC ACC AGA AAC ATG CCG GAT ATT GCT AAT GTA CCT
AAT TTC AAC ACT GTC GGT GGC GGA ATA GAC TAT ATG TTC AAA GAT AAG ATT GGT
GCA TCT GCG AGC GCG GCT CAC ACG GAC TTT ATC AAT CGC AAC GAC TAC TCT CTT
GAC GCG AAA CTG AAC CTC TTC AAG ACT CCT GAT ACC TCG ATT GAT TTC AAC GCC
GGT TTC AAG AAG TTC GAT ACA CCT TTC ATG AAG TCC TCT TGG GAG CCT AAC TTC
GGA TTC TCA CTT TCT AAA TAT TTC TGA.

SEQUENCE LISTING

-42-

28. The process of Claim 13 in which said antimicrobial agent is an E protein from phage PhiX174 and said transformed animal cells contain the gene sequence as follows:

ATGGT ACGCTGGACT TTGTGGGATA CCCTCGCTTT CCTGCTCCTG TTGAGTTTAT
TGCTGCCGTC ATTGCTTATT ATGTTTCATCC CGTCATTCAAACG GCCTGTCTCA
TCATGGAAGG CGCTGAATTT ACGGAAAACA TTATTAATGG CGTCGAGCGT
CCGGTTAAAG CCGCTGAATT GTTCGCGTTT ACCTTGCGTG TACGCGCAGG
AAACACTGAC GTTCTTACTG ACCCAGAAGA AAACGTGCGT CAAAATTAC
GTGCGGAAGG AGTGA.

29. The process of Claim 13 in which said antimicrobial agent is a lysozyme and said transformed animal cells contain the gene sequence as follows:

AGATCTGTTTCATG AAA CGT TTC ACG AGA TGC GGG TTA GTG CAG GAG CTT AGG AGA CGA GGC
TTC GAT GAA ACT TTG ATG AGT AAC TCG GTC TGC CTT GTC GAG AAC GAA AGC GGA CGG TTT ACC
GAT AAA ATC GGT AAA GTT AAC AAG AAC GGA TGT CGA GAC TAC GGC CTC TTC CAG ATC AAT GAC
AAA TAC TCG TCG AGT AAG GGA TCC ACT CCT GGA AAG GAT TGC AAC GTG ACT TGT AAT CAG CTA
CTG ACT GAC GAC ATT AGC GTG GCA GCT ACG TCG GCG AAG AAG ATT TAC AAA CGC CAC AAG TTT
GAC GCT TGG TAC GGA TGG AAA AAT CAC TGT CAA CAT GGA CTG CCA GAT ATT AGC GAC TGT TAG
AGACGACTTATTATAGCCTTC GTTTCATGAAAC AGATCT.

30. The process of Claim 13 in which said antimicrobial agent is an S protein from lambda phage and said transformed animal cells contain the gene sequence as follows:

ATGAA GATGCCAGAA AAACATGACC TGTGCGGCCG CCATTCTCGC GGCAAAGGAA
CAAGGCATCG GGGCAATCCTT GCGTTTGCAA TGGCGTACCT TCGCGGCAGA
TATAATGGCG GTGCGTTTAC AAAACAGTA ATCGACGCAA CGATGTGCGC
CATTATCGCC TGGTTCATTC GTGACCTTCT CGACTTCGCC GGACTAAGTA
GCAATCTCGC TTATATAACG AGCGTGTTTA TCGGCTACAT CGGTACTGAC

SUBSTITUTE SHEET

TCGATTGGTT CGCTTATCAA ACGCTTCGCT GCTAAAAAAG CCGGAGTAGA
AGATGGTAGA AATCAATAA.

31. The process of Claim 13 in which said antimicrobial agent is a protein produced by gene 13 of phage 22 and said transformed animal cells contain the following gene sequence:

ATGAA AAAGATGCCA GAAAAACATG ATCTGTTAAC CGCCATGATG GCGGCAAAGG
AACAGGGCAT CGGGGCAATC CTCGCGTTTG CAATGGCGTA CCTTCGCGGT
CGGTATAATG GCGGTGCGTT TAAGAAAACA CTAATAGACG CAACGATGTG
CGCCATTATC GCCTGGTTCA TTCGTGACCT TTAGTCTTC GCCGGACTGA
GTAGCAATCT TGCTTACATA GCGAGTGTGT TTATCGGCTA CATCGGCACA
GACTCGATTG GTTCGCTAAT CAAACGCTTC GCTGCTAAAA AAGCCGGAGT
CGATGATGCA AATCAGCAGT AA.

32. A process for providing animals with resistance to microbial infections comprising:

(a) transforming animal cells with one or more genes coding for one or more antimicrobial agents selected from the group consisting of cecropins, attacins, lysozymes, an S protein from lambda phage, an E protein from phage PhiX174, and a protein produced by gene 13 of phage 22, such that said cells are capable of expressing said one or more antimicrobial agents; and

(b) implanting said transformed cells into said animal, such that said implanted transformed cell expresses said one or more antimicrobial agents.

33. The process of Claim 32 wherein said animal cells are selected from fibroblast cells, macrophage cells, primary cells, pluripotent embryonic stem cells, pluripotent

-44-

hematopoietic stem cells, phagocytic cells, plasma cells, mast cells and carcinoma cells.

34. The process of Claim 32 in which said animal cells are transformed by

- (a) treatment with chemical transformants,
- (b) treatment with a modified retrovirus vector, or
- (c) treatment employing electroporation.

35. The process of Claim 34 in which said animal cells are transformed by treatment with a chemical transformant.

36. The process of Claim 34 in which said chemical transformant is selected from calcium phosphate and dextran sulfate.

37. The process of Claim 35 in which said chemical transformant is calcium phosphate.

38. The process of Claim 34 in which said animal cells are transformed by treatment with a modified retrovirus vector.

39. The process of Claim 38 in which said modified retrovirus vector is selected from the group consisting of N2, N4, SAX, Mo+Py, and M-MuLV.

40. The process of Claim 39 in which said modified retrovirus vector is N2.

41. The process of Claim 34 in which said animal cells are transformed by electroporation.

SUBSTITUTE SHEET

-45-

42. The process of Claim 32 in which said antimicrobial agent is a cecropin.

43. The process of Claim 42 in which said cecropin is selected from cecropin A, cecropin B, and a modified cecropin B having methionine and proline added at the amino end.

44. The process of Claim 43 in which said cecropin is Cecropin B and said transformed animal cells contain the gene sequence as follows:

AAATGCAAAGTCTTCAAGAAA ATT GAA AAA ATG GGT CGC AAC ATT CGA AAC GGT ATT GTC AAG
GCT CGA CCA GCG ATC GCG GTT TTA GGC GAA GCC AAA GCG CTA GGA TAA.

45. The process of Claim 43 in which said antimicrobial agent is a modified cecropin B and contains the gene sequence as follows:

ATGCCGAAATGGAAAGTCTTCAAGAAAATT GAA AAA GTC GGT CGC AAC ATT CGA AAC GGT ATT GTC
AAG GCT CGA CCA GCG ATC GCG GTT TTA GGC GAA GCC AAA GCG CTA GGA TAA.

46. The process of Claim 32 in which said antimicrobial agent is an attacina and said cell has the gene sequence as follows:

ATG
GAC GCG CAC GGA GCC CTT ACG CTC AAC TCC GAT GGT ACC TCT GGT GCT GTG GTT
AAA GTA CCC TTT CGT GGT AAC GAC AAG AAT ATA GTA AGC GCT ATC GGT TCC GTA
GAC TTA ACT GAT AGG CAG AAA CTA GGC GCT GCA ACC GCT GGA GTG GCA CTG GAT
AAT ATA AAC GGT CAC GGA CTA AGT CTC ACG GAT ACA CAC ATC CCC GGG TTC GGA
GAC AAG ATG ACA GCA GCC GGC AAA GTC AAT GTG TTC CAC AAT GAT AAC CAC GAC

SUBSTITUTE SHEET

-46-

ATC ACA GCG AAG GCT TTC GCC ACC AGA AAC ATG COG GAT ATT GCT AAT GTA CCT
AAT TTC AAC ACT GTC GGT GGC GGA ATA GAC TAT ATG TTC AAA GAT AAG ATT GGT
GCA TCT GCG AGC GCC GCT CAC ACG GAC TTT ATC AAT CGC AAC GAC TAC TCT CTT
GAC GGG AAA CTG AAC CTC TTC AAG ACT CCT GAT AGC TCG ATT GAT TTC AAC GCC
GGT TTC AAG AAG TTC GAT ACA CCT TTC ATG AAG TCC TCT TGG GAG CCT AAC TTC
CGA TTC TCA CTT TCT AAA TAT TTC TGA.

47. The process of Claim 32 in which said antimicrobial agent is a lysozyme and said cell has the gene sequence as follows:

AGATCTGTTTCATG AAA CGT TTC ACG AGA TGC GGG TTA GTG CAG GAG CTT AGC AGA CGA GGC
TTC GAT GAA ACT TTG ATG AGT AAC TGG GTC TGC CTT GTC GAG AAC GAA AGC GGA CGG TTT ACC
GAT AAA ATC GGT AAA-GTT AAC AAG AAC GGA TCT CGA GAC TAC GGC CTC TTC CAG ATC AAT GAC
AAA TAC TGG TGC AGT AAG GGA TCC ACT CCT GGA AAG GAT TGC AAC GTG ACT TGT AAT CAG CTA
CTG ACT GAC GAC ATT AGC GTG GCA GGT ACG TGC GCG AAG AAG ATT TAC AAA CGC CAC AAG TTT
GAC GCT TGG TAC GGA TGG AAA AAT CAC TGT CAA CAT GGA CTG CCA GAT ATT AGC GAC TGT TAG
AGACGACTTATTATAGCCTTC GTTTCATGAAAC AGATCT.

48. The process of Claim 32 in which said antimicrobial agent is an E protein from phage PhiX174 and contains the gene sequence as follows:

ATGGT ACGCTGGACT TTGTGGGATA CCCTCGCTTT CCTGCTCCTG TTGAGTTTAT
TGCTGCCGTC ATTGCTTATT ATGTTTCATCC CGTCATTCAAACG GCCTGTCTCA
TCATGGAAGG CGCTGAATTT ACGGAAAACA TTATTAATGG CGTCGAGCGT
CCGGTTAAAG CCGCTGAATT GTTCGCGTTT ACCTTGCGTG TACGCGCAGG
AAACACTGAC GTTCTTACTG ACGCAGAAGA AAACGTGCGT CAAAATTAC
GTGCGGAAGG AGTGA.

SUBSTITUTE SHEET

-47-

49. The process of Claim 32 in which said antimicrobial agent is an S protein from lambda phage and contains the gene sequence as follows:

ATGAA GATGCCAGAA AAACATGACC TGTGGGGCCG CCATTCTCGC GGCAAAGGAA
CAAGGCATCG GGGCAATCCTT GCGTTTGCAA TGGCGTACCT TCGCGGCAGA
TATAATGGCG GTGCGTTTAC AAAAACAGTA ATCGACGCAA CGATGTGCGC
CATTATCGCC TGGTTCATTC GTGACCTTCT CGACTTCGCC GGACTAAGTA
GCAATCTCGC TTATATAACG AGCGTGTTTA TCGGCTACAT CGGTACTGAC
TCGATTGGTT CGCTTATCAA ACGCTTCGCT GCTAAAAAAG CCGGAGTAGA
AGATGGTAGA AATCAATAA.

50. The process of Claim 32 in which said antimicrobial agent is a protein produced by gene 13 of phage 22 and contains the following gene sequence:

ATGAA AAAGATGCCA GAAAACATG ATCTGTTAAC CGCCATGATG GCGGCAAAGG
AACAGGGCAT CGGGGCAATC CTCGCGTTTG CAATGGCGTA CCTTCGCGGT
CGGTATAATG GCGGTGCGTT TAAGAAAACA CTAATAGACG CAACGATGTG
CGCCATTATC GCCTGGTTCA TTCGTGACCT TTAGTCTTC GCCGGACTGA
GTAGCAATCT TGCTTACATA GCGAGTGTGT TTATCGGCTA CATCGGCACA
GACTCGATTG GTTCGCTAAT CAAACGCTTC GCTGCTAAAA AAGCCGGAGT
CGATGATGCA AATCAGCAGT AA.

51. The process of Claim 32 in which said animals are mammals.

52. The process of Claim 32 in which said mammals are selected from cattle, pigs, sheep, horses, goats, dogs, cats, and human beings.

ORIGINAL SHEET

-48-

53. The process of Claim 52 in which said mammals are human beings.

54. The process of Claim 52 in which said mammals are cattle.

55. The process of Claim 52 in which said mammals are pigs.

56. The process of Claim 52 in which said mammals are sheep.

57. The process of Claim 52 in which said mammals are horses.

58. The process of Claim 52 in which said mammals are goats.

59. The process of Claim 52 in which said mammals are dogs.

60. The process of Claim 52 in which said mammals are cats.

61. A process for producing embryonic animal cells having in their genomes genes coding for one or more antimicrobial agents selected from the group consisting of cecropins, attacins, lysozymes, an S protein from lambda phage, an E protein from phage PhiX174 and a protein produced by gene 13 of phage 22, comprising transforming said cells with said genes, wherein said transformation is accomplished by:

SUBSTITUTE SHEET

-49-

(a) microinjection of said embryonic animal cells with one or more genes coding for said antimicrobial agents;

(b) transfection of said embryonic animal cells with retroviral vectors carrying one or more genes coding for said antimicrobial agents;

(c) electroporation of said embryonic animal cells with one or more genes coding for said antimicrobial agents in a culture medium solution; or

(d) treatment of said embryonic animal cells with a chemical transformant solution containing one or more genes coding for said antimicrobial agents.

62. The process of Claim 61 in which said embryonic animal cells are early preimplantation stage embryos selected from pronuclear stage embryos, one-cell embryos, 2-cell embryos, 4-cell embryos, 8-cell embryos, morulae and blastocysts.

63. The process of Claim 61 in which said transformation is accomplished by microinjection.

64. The process of Claim 63 in which said embryonic animal cell is a pronuclear stage embryo.

65. The process of Claim 61 in which said transformation is accomplished by transfection of said embryonic animal cell with a retroviral vector.

66. The process of Claim 65 in which said early preimplantation stage embryos are selected from pronuclear

SUBMITTED

-50-

stag embryo s, one-cell embryos, 2-cell embryos, 4-cell embryos, 8-cell embryos, morulae and blastocysts.

67. The process of Claim 66 in which said retroviral vector is selected from the group consisting of N2, N4, SAX, Mo+Py and M-MuLV.

68. The process of Claim 67 in which said retroviral vector is N2.

69. The process of Claim 61 in which said transformation is accomplished by electroporation.

70. The process of Claim 69 in which said embryonic animal cells are early preimplantation embryos.

71. The process of Claim 61 in which said transformation is accomplished by treatment of said embryonic animal cells with a chemical transformant.

72. The process of Claim 71 in which said chemical transformant is selected from calcium phosphate and dextran sulfate.

73. The process of Claim 72 in which said chemical transformant is calcium phosphate.

74. The process of Claim 61 wherein one of said genes codes for a cecropin antimicrobial agent and has the gene sequence as follows:

-51-

AAATGGAAAGTCTTCAAGAAA ATT GAA AAA ATG GGT CGC AAC ATT CGA AAC GGT ATT GTC AAG
GCT GGA CCA GCG ATC GCG GTT TTA GGC GAA GCC AAA GCG CTA GGA TAA

75. The process of Claim 61 wherein one of said genes codes for an acidic attacin antimicrobial agent and has the gene sequence as follows:

ATG

GAC GCG CAC GGA GCC CTT ACG CTC AAC TCC GAT GGT ACC TCT GGT GCT GTG CTT
AAA GTA CCC TTT CGT GGT AAC GAC AAG AAT ATA GTA AGC GCT ATC GGT TCC GTA
GAC TTA ACT GAT AGG CAG AAA CTA GGC GCT GCA ACC GCT GGA GTG GCA CTG GAT
AAT ATA AAC GGT CAC GGA CTA AGT CTC ACG GAT ACA CAC ATC CCC GGG TTC GGA
GAC AAG ATG ACA GCA GCC GGC AAA GTG AAT GTC TTC CAC AAT GAT AAC CAC GAC
ATC ACA GCG AAG GCT TTC GCC ACC AGA AAC ATG CCG GAT ATT GCT AAT GTA CCT
AAT TTC AAC ACT GTC GGT GGC GGA ATA GAC TAT ATG TTC AAA GAT AAG ATT GGT
GCA TCT GCG AGC GCC GCT CAC ACG GAC TTT ATC AAT CGC AAC GAC TAC TCT CTT
GAC GGG AAA CTG AAC CTC TTC AAG ACT CCT GAT ACC TCG ATT GAT TTC AAC GCC
GGT TTC AAG AAG TTC GAT ACA CCT TTC ATG AAG TCC TCT TGG GAG CCT AAC TTC
GGA TTC TCA CTT TCT AAA TAT TTC TGA

76. The process of Claim 61 wherein one of said genes codes for a lysozyme antimicrobial agent and has the gene sequence as follows:

AGATCTGTTTCATG AAA CGT TTC ACG AGA TGG GGG
TTA GTG CAG GAG CTT AGG AGA CGA GGC TTC GAT GAA ACT TTG ATG AGT AAC TGG
GTC TGC CTT GTC GAG AAC GAA AGC GGA CCG TTT ACC GAT AAA ATC GGT AAA GTT
AAC AAG AAC GGA TCT CGA GAC TAC GGC CTC TTC CAG ATC AAT GAC AAA TAC TGG
TGC AGT AAG GGA TCC ACT CCT GGA AAG GAT TGC AAC GTG ACT TGT AAT CAG CTA
CTG ACT GAC GAC ATT AGC GTG GCA GCT ACG TGC GCG AAG AAG ATT TAC AAA CGC
CAC AAG TTT GAC GCT TGG TAC GGA TGG AAA AAT CAC TGT CAA CAT GGA CTG CCA
GAT ATT AGC GAC TGT TAG AGACCACTTATTATAGCCTTC GTTTCATGAAAC AGATCT.

SUBSTITUTE SHEET

-52-

77. The process of Claim 61 wherein one of said genes codes for an S protein from lambda phage and has the gene sequence as follows:

ATGAA GATGCCAGAA AAACATGACC TGTGGGCGG CCATTCTCGC GGCAAAGGAA
CAAGGCATCG GGGCAATCCTT GCGTTTGCAA TGGCGTACCT TCGCGGCAGA
TATAATGGCG GTGCGTTTAC AAAACAGTA ATCGACGCAA CGATGTGCGC
CATTATCGCC TGGTTCATTC GTGACCTTCT CGACTTCGCC GGACTAAGTA
GCAATCTCGC TTATATAACG AGCGTGTTTA TCGGCTACAT CCGTACTGAC
TCGATTGGTT CGCTTATCAA ACGCTTCGCT GCTAAAAAAG CCGGAGTAGA
AGATGGTAGA AATCAATAA.

78. The process of Claim 61 wherein one of said genes codes for an E protein from phage PhiX174 and has the gene sequence as follows:

ATGGT ACGCTGGACT TTGTGGGATA CCCTCGCTTT CTGCTCCTG TTGAGTTTAT
TGCTGCCGTC ATTGCTTATT ATGTTTCATCC CGTCATTCAAACG GCCTGTCTCA
TCATGGAAGG CGCTGAATTT ACGGAAAACA TTATTAATGG CGTCGAGCGT
CCGGTTAAAG CCGCTGAATT GTTCGCGTTT ACCTTGCGTG TACGCGCAGG
AAACACTGAC GTTCTTACTG ACGCAGAAGA AAACGTGCGT CAAAATTAC
GTGCGGAAGG AGTGA.

79. The process of Claim 61 wherein one of said genes codes for a protein produced from gene 13 of phage 22 and has the gene sequence as follows:

ATGAA AAAGATGCCA GAAAAACATG ATCTGTTAAC CGCCATGATG GCGGCAAAGG
AACAGGGCAT CGGGGCAATC CTCGCGTTTG CAATGGCGTA CCTTCGCGGT
CGGTATAATG GCGGTGCGTT TAAGAAAACA CTAATAGACG CAACGATGTG
CGCCATTATC GCCTGGTTCA TTCGTGACCT TTAGTCTTC GCCGGACTGA

-55-

GTAGCAATCT TGCTTACATA GCGAGTGTGT TTATCGGCTA CATCGGCACA
GACTCGATTG GTTCGCTAAT CAAACGCTTC GCTGCTAAAA AAGCCGGAGT
CGATGATGCA AATCAGCAGT AA.

80. A process for producing an animal resistant to microbial disease which comprises the steps of

(a)(i) implanting a mammalian transformed embryo into the womb of a suitable mother animal, (ii) culturing the transformed eggs of aquatic species, or (iii) incubating transformed fowl eggs which transformation incorporates into the genome one or more genes coding for one or more antimicrobial agents according to the process of Claim 61 into the womb of a suitable mother animal,

(b) allowing normal development and birth or hatching, as appropriate,

whereby an animal resistant to microbial disease is produced.

81. An animal having in its genome one or more genes coding for one or more antimicrobial agents selected from the group consisting of cecropins, attacins, lysozymes, an S protein from lambda phage, an E protein from phage PhiX174, and a protein from gene 13 of phage 22, such that said animal is resistant to microbial diseases.

82. The animal of Claim 81 selected from the group consisting of horses, cattle, pigs, sheep, goats, dogs, cats, fowl and aquatic species.

83. The animal of Claim 81 in which said animal is resistant to microbial diseases caused by an ordinarily disease-producing amount of a microbe selected from Brucella,

SUBSTITUTE SHEET

Listeria, Pseudomonas, Staphyl coccus, Trypan some and Plasmodium.

84. The animal of Claim 83 wherein said microbe is Brucella.

85. The animal of Claim 84 wherein said Brucella is Brucella abortus.

86. The animal of Claim 83 wherein said microbe is Plasmodium.

87. The animal of Claim 86 wherein said microbe is Plasmodium falciparum.

88. The animal of Claim 83 wherein said microbe is Trypanosoma.

89. The animal of Claim 88 wherein said microbe is Trypanosoma cruzi.

90. The animal of Claim 83 wherein said microbe is Listeria.

91. The animal of Claim 90 wherein said Listeria is Listeria monocytogenes.

92. The animal of Claim 83 wherein said microbe is Pseudomonas.

93. The animal of Claim 83 wherein said Pseudomonas is Pseudomonas aeruginosa.

-55-

94. The animal of Claim 83 wherein said microbe is Staphylococcus.

95. The animal of Claim 94 wherein said Staphylococcus is Staphylococcus aureus.

96. The animal of Claim 81 in which said one or more genes coding for said one or more antimicrobial agents codes for a cecropin and has the gene sequence as follows:

AAATGGAAAGTCTTCAAGAAA ATT GAA AAA ATG GGT CGC AAC ATT CGA AAC CGT ATT CTC AAG
GCT GGA CCA GCG ATC GCG GTT TTA GGC GAA GCC AAA GCG CTA GGA TAA.

97. The animal of Claim 81 in which said one or more genes coding for said one or more antimicrobial agents codes for an acidic attacin and has the gene sequence as follows:

ATG
GAC GCG CAC GGA GCC CTT ACG CTC AAC TCC GAT GGT ACC TCT GGT GCT GTC GTT
AAA GTA CCC TTT CGT GGT AAC GAC AAG AAT ATA GTA AGC GCT ATC GGT TCC GTA
GAC TTA ACT GAT AGG CAG AAA CTA GGC GCT GCA ACC GCT GGA GTG GCA CTG GAT
AAT ATA AAC GGT CAC GGA CTA AGT CTC ACG GAT ACA CAC ATC CCC GGG TTC GGA
GAC AAG ATG ACA GCA GCC GGC AAA GTG AAT GTC TTC CAC AAT GAT AAC CAC GAC
ATC ACA GCG AAG GCT TTC GCC ACC AGA AAC ATG CCG GAT ATT GCT AAT GTA CCT
AAT TTC AAC ACT GTC GGT GGC GGA ATA GAC TAT ATG TTC AAA GAT AAG ATT GGT
GCA TCT GCG AGC GCC GCT CAC ACG GAC TTT ATC AAT CGC AAC GAC TAC TCT CTT
GAC GGG AAA CTG AAC CTC TTC AAG ACT CCT GAT ACC TCG ATT GAT TTC AAC GCC
GGT TTC AAG AAG TTC GAT ACA CCT TTC ATG AAG TCC TCT TGG GAG CCT AAC TTC
GGA TTC TCA CTT TCT AAA TAT TTC TGA.

SUBSTITUTE SHEET

-56-

98. The animal of Claim 81 in which said one or more genes coding for said one or more antimicrobial agents codes for a lysozyme and has the gene sequence as follows:

AGATCTGTTTGATG AAA CGT TTC ACG AGA TGC GGG
TTA GTG CAG GAG CTT AGG AGA CGA GGC TTC GAT GAA ACT TTG ATG AGT AAC TGG
GTC TGC CTT GTC GAG AAG GAA AGC GGA CGG TTT ACC GAT AAA ATC GGT AAA GTT
AAC AAG AAC GGA TCT CGA GAC TAC GGC CTC TTC CAG ATC AAT GAC AAA TAC TGG
TGC AGT AAG GGA TCC ACT CCT GGA AAG GAT TGC AAC GTG ACT TGT AAT CAG CTA
CTG ACT GAC GAC ATT AGC GTG GCA GCT ACG TGC GCG AAG AAG ATT TAC AAA CGC
CAC AAG TTT GAC GCT TGG TAC GGA TGG AAA AAT CAG TGT CAA CAT GGA CTG CCA
CAT ATT ACC GAC TGT TAG AGACGACTTATTATAGCCTTC GTTTCATGAAAC AGATCT.

99. The animal of Claim 81 in which said one or more genes coding for an S protein from lambda phage and has the gene sequence as follows:

ATGAA GATGCCAGAA AAACATGACC TGTTGGGCCG CCATTCTCGC GGCAAAGGAA
CAAGGCATCG GGGCAATCCTT GCGTTTGCAA TGGCGTACCT TCGCGGCAGA
TATAATGGCG GTGCGTTTAC AAAAACAGTA ATCGACGCAA CGATGTGCGC
CATTATCGCC TGGTTCATTC GTGACCTTCT CGACTTCGCC GGACTAAGTA
GCAATCTCGC TTATATAACG AGCGTGTTTA TCGGCTACAT CGGTACTGAC
TCGATTGGTT CGCTTATCAA ACGCTTCGCT GCTAAAAAAG CCGGAGTAGA
AGATGGTAGA AATCAATAA.

100. The animal of Claim 81 in which said one or more genes coding for an E protein from phage PhiX174 and has the gene sequence as follows:

ATGGT ACGCTGGACT TTGTGGGATA CCCTCGCTTT CTGCTCCTG TTGAGTTTAT
TGCTGCCGTC ATTGCTTATT ATGTTTCATCC CGTCATTCAAACG GCCTGTCTCA
TCATGGAAGG CGCTGAATTT ACGGAAAACA TTATTAATGG CGTCGAGCGT

END OF PAGE SHEET

-57-

CCGGTTAAAG CCGCTGAATT GTTCGCGTTT ACCTTGCGTG TACGCGCAGG
AAACACTGAC GTTCTTACTG ACGCAGAAGA AAACGTGCGT CAAAATTAC
GTGCGGAAGG AGTGA.

101. The animal of Claim 81 in which said one or more genes coding for a protein produced from gene 13 of phage 22 and has the gene sequence as follows:

ATGAA AAAGATGCCA GAAAACATG ATCTGTTAAC CGCCATGATG GCGGCAAAGG
AACAGGGCAT CGGGGCAATC CTCGCGTTTG CAATGGCGTA CCTTCGCGGT
CGGTATAATG GCGGTGCGTT TAAGAAAACA CTAATAGACG CAACGATGTG
CGCCATTATC GCCTGGTTCA TTCGTGACCT TTAGTCTTC GCCGGACTGA
GTAGCAATCT TGCTTACATA GCGAGTGTGT TTATCGGCTA CATCGGCACA
GACTCGATTG GTTCGCTAAT CAAACGCTTC GCTGCTAAA AAGCCGGAGT
CGATGATGCA AATCAGCAGT AA.

102. A method of treatment of humans and animals for microbial infections comprising administering to said animals an antimicrobially effective amount of one or more antimicrobial agents selected from the group consisting of a cecropin, an attacin, a lysozyme, an S protein from lambda phage, an E protein from phage PhiX174, and a protein produced by gene 13 of phage 22.

103. The method of Claim 102 in which said microbial infection is selected from Brucellosis, malarial infection, antibiotic-resistant infections, Listeriosis and Chagas' disease.

104. The method of Claim 102 in which said antimicrobial agent is a cecropin.

SUBSTITUTE SHEET

-58-

105. The method of Claim 104 in which said cecropin is selected from cecropin A, cecropin B, modified cecropin B having methionine and proline added at the amino end and free from internal methionine.

106. The method of Claim 105 in which said cecropin is Cecropin B and having the amino acid sequence as follows:

LysTrpLysValPheLysLysIleGluLysMetGlyArgAsnIleArgAsnGlyIleVal
LysAlaGlyProAlaIleAlaValLeuGlyGluAlaLysAlaLeuCONH₂.

107. The method of Claim 105 in which said antimicrobial agent is a cecropin B which is modified by having 37 amino acid sequence including methionine and proline added at the amino end as follows:

MetProLysTrpLysValPheLysLysIleGluLysValGlyArgAsnIleArgAsnGly
IleValLysAlaGlyProAlaIleAlaValLeuGlyGluAlaLysAlaLeuCONH₂.

108. The method of Claim 102 in which said antimicrobial agent is a truncated E protein from phage PhiX174 having the amino acid sequence as follows:

MetValArgTrpThrLeuTrpAspThrLeuAlaPheLeuLeuLeuLeuSerLeuLeuLeu
ProSerLeuLeuIleMetPheIleProSerPheLysArgProVal.

109. The method of Claim 102 in which said antimicrobial agent is an S protein from lambda phage having the amino acid sequence as follows:

-59-

MetLysMet ProGluLysHisAspLeuLeuAlaAlaIleLeuAlaAlaLysGluGluGly
IleGlyAlaIleLeuIlePheAlaMetAlaTyrLeuArgGlyArg.

110. The method of Claim 102 in which said antimicrobial agent is a protein produced by truncated gene 13 of phage 22 in which the first thirty amino acids have the following sequence:

MetLysLysMetProGluLysHisAspLeuLeuThrAlaMetMetAlaAlaLysGluGln
GlyIleGlyAlaIleLeuAlaPheAlaMetAlaTyrLeuArgGlyArg.

111. A medicinal composition for treatment of humans and animals for microbial infections comprising an antimicrobially effective amount of one or more antimicrobial agents selected from the group consisting of a cecropin, an attacin, a lysozyme, an S protein from lambda phage, an E protein from phage PhiX174 and a protein produced by gene 13 of phage 22 and a pharmaceutically acceptable carrier.

112. The composition of Claim 111 in which said microbial infection is selected from Brucellosis, malarial infection, antibiotic-resistant infections, Listeriosis and Chagas' disease.

113. The composition of Claim 111 in which said antimicrobial agent is a cecropin.

114. The composition of Claim 113 in which said cecropin is selected from cecropin A, cecropin B, modified cecropin B having methionine and proline added at the amino end and free from internal methionine.

-60-

115. The composition of Claim 114 in which said cecropin is Cecropin B and having the amino acid sequence as follows:

LysTrpLysValPheLysLysIleGluLysMetGlyArgAsnIleArgAsnGlyIleVal
LysAlaGlyProAlaIleAlaValLeuGlyGluAlaLysAlaLeuCONH₂.

116. The composition of Claim 114 in which said antimicrobial agent is a cecropin B which is modified by having 37 amino acid sequence including methionine and proline added at the amino end as follows:

MetProLysTrpLysValPheLysLysIleGluLysValGlyArgAsnIleArgAsnGly
IleValLysAlaGlyProAlaIleAlaValLeuGlyGluAlaLysAlaLeuCONH₂.

117. The composition of Claim 111 in which said antimicrobial agent is a truncated E protein from phage PhiX174 having the amino acid sequence as follows:

MetValArgTrpThrLeuTrpAspThrLeuAlaPheLeuLeuLeuLeuSerLeuLeuLeu
ProSerLeuLeuIleMetPheIleProSerPheLysArgProVal.

118. The composition of Claim 111 in which said antimicrobial agent is a truncated S protein from lambda phage having the amino acid sequence as follows:

MetLysMet ProGluLysHisAspLeuLeuAlaAlaIleLeuAlaAlaLysGluGluGly
IleGlyAlaIleLeuIlePheAlaMetAlaTyrLeuArgGlyArg.

119. The method of Claim 111 in which said antimicrobial agent is a protein produced by truncated gene 13 of phage 22 in which the first thirty amino acids have the following sequence:

MetLysLysMetPr GluLysHisAspLeuLeuThrAlaMetMetAlaAlaLysGluGln
GlyIleGlyAlaIleLeuAlaPheAlaMetAlaTyrLeuArgGlyArg.

INTERNATIONAL SEARCH REPORT

International Application

PCT/US88/02265

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. CL.: 435/172.3

INT. CL.: (4) C12N.15/00

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.	435/68, 70, 172.3, 240.2, 320 800/1, 530/350,858 935/11, 32, 53, 63

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

Chemical Abstracts, Biological Abstracts

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	US, A, 4,579,821 (PALMITER) 01 April 1986, See abstract.	1-101
X Y	US, A, 4,520,016 (HULTMARK) 28 May 1985, See column 8 lines 4-14.	102-107, 111-116 1-101, 108-110 117-119
Y	Proceedings of the National Academy of Sciences, 82 December 1985, Huszar et al. "Insertion of a Bacterial Gene into the Mouse Germ Line Using an Infectious Retrovirus Vector", pages 8587-91.	19-31

⁹ Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

17 October 1988

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

30 NOV 1988

Signature of Authorized Officer

JOHN TARCZA

John E. Tarcza

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X A	Chemical Abstracts, Volume 106, No. 23, issued 8 June 1987, (Columbus, Ohio, USA) Lopez et al. Production of Lysozyme of Streptococcus Pneumonia in Escherichia coli by Recombinant DNA Technology" see page 207, abstract no. 190368d, Spanish 545 689, 01 May 1986).	111,112 29,76,98
Y	Molecular and General Genetics, 182, 1981, Garrett et al, "Cell Lysis by Induction of Cloned Lambda Lysis Genes", pages 321-31.	30,77, 99,109, 118
Y	EMBO Journal 3(9), 1984, Kockum- et al, "Insect Immunity, Isolation and Sequence of Two cDNA clones Corresponding to Acidic And Basic Attacins from Hyalophora Cecropia", pages 2071-5.	27,75 97
Y	Proceedings of the National Academy of Sciences, 82, April 1985, Hofsten et al, "Molecular Cloning, cDNA Sequencing and Chemical Synthesis of Cecropin B from Hyalophora Cecropia". page 2240-3.	4-7, 23-26, 74,96, 104-107 and 113-116
Y	Journal of General Virology, 66, 1985, Blasi et al, "Influence of C-Terminal Modifications of ϕ x174 Lysis Gene E on its Lysis Inducing Properties", pages 1209-13.	28,78, 100, 108 and 117
Y	Virology, 143, 1985, Rennell et al "Phage P22 Lysis Genes", Nucleotide Sequence and Functional Relationships with T4 and λ Genes, pages 280-9.	81,79, 101,110, and 119